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Report

# Actin Depolymerization-Based Force Retracts the Cell Rear in Polarizing and Migrating Cells

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## Summary

**In migrating cells, the relative importance of myosin II contractility for cell rear retraction varies [1–12]. However, in myosin II-inhibited polarizing cells, actin organization is compromised [13–18]; thus it remains unclear whether myosin II is simply required for correct actin arrangement or also directly drives rear retraction [9]. Ascaris sperm cells lack actin and associated motors, and depolymerization of major sperm protein is instead thought to pull the cell rear forward [19, 20]. Opposing views exist on whether actin could also have this function [19, 20] and has not been directly experimentally sought. We probe function at high temporal resolution in polarizing fibroblasts that establish migration by forming the cell rear first [9, 15, 21]. We show that in cells with correctly organized actin, that actin filament depolymerization directly drives retraction of the rear margin to polarize cells and spatially accounts for most cell rear retraction during established migration. Myosin II contractility is required early, to form aligned actin bundles that are needed for polarization, and also later to maintain bundle length that ensures directed protrusion at the cell front. Our data imply a new mechanism: actin depolymerization-based force retracts the cell rear to polarize cells with no direct contribution from myosin II contractility.**

## Results

Cell polarization and triggering of migration—the process where a single cell front and cell rear form at opposite ends of the cell—is complex, involving a number of distinct identified stages that occur stepwise with time [9, 15, 21] (see Figure S1 available online). The breaking of cell symmetry is defined as the first shape change event that results in cell asymmetry. We define temporal analysis of function as acute probing of a given protein's activity right at a particular identified stage of cell polarization.

Because RNA interference (RNAi)-based systems, for example, are too slow for temporal analysis of function, we validated that blebbistatin, a small chemical inhibitor that specifically blocks myosin II ATPase [22], is suitable for such analysis at sufficient resolution in primary chick heart fibroblasts (see Supplemental Information and Figure S2). Taken together, blebbistatin rapidly targets, within 1 min, myosin II contractility right at the cell retraction step in chick fibroblasts without also compromising correct actin organization at this stage.

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### Temporal Analysis One of Myosin II Contractility Function

To mark when cells are going to form the cell rear to break cell symmetry, we used as an indicator the known timing of alignment of graded polarity actomyosin II filament bundles in live synchronized, polarizing fibroblasts because this is the actin organization that triggers polarization in these cells [21] (Figure S1) (see also Supplemental Experimental Procedures).

Treatment of cells with 100 μM (±) (50 μM active concentration) blebbistatin before actomyosin II bundles had aligned blocked establishment of polarity (Figure 1A); no single cell front or cell back resolved (Figure 1, compare B with C) (Movie S1, compare clips 1 and 2) in a time of observation by which untreated cells had fully polarized (Figure 1B).

In contrast, addition of 100 μM (±) blebbistatin to cells after actomyosin II bundles were expected to have aligned and prior to expected cell margin retraction did not block establishment of polarity (Figure 1A, compare B with D, black arrows); a single cell rear resolved, initially normally (Figures 1A and 1D; 19 min) (Movie S1, clip 3) at a similar rate as control cells (Figure 1E, 0–20 min). In this treatment, oriented actin filament bundles were expected to be mostly intact (see Supplemental Information). As an additional test that blebbistatin was active, speed of cell body translocation in the direction of migration, which is driven by myosin II in these [23] and other cells [24, 25] (Movie S2 in [22]), was reduced more than 3-fold in the same individual cell treated with blebbistatin and at the same time as when establishment of the cell rear was unaffected by the same treatment (compare cell body position in Figure 1B with D).

### Spatial Analysis of Rear Retraction Speed

It became evident, however, that as the same recently polarized cell (Figure 1D, 19 min) started to migrate that there was then a difference in rear retraction speed, dependent on spatial location along the same rear margin. Retraction of most spatial zones along the cell rear in migrating cells appeared independent of myosin II-based force (Figures 1D–1G, 19–40 min) (Movie S1, clips 3 and 4), moving at a similar speed to untreated cells (Figure 1E, 19–40 min). However, forward translocation of the tip of the cell rear (Figure 1F, black arrowhead) and a few other isolated zones, reminiscent of retraction fibers, required myosin II contractility, moving 1/3 slower or less than controls (Figures 1E and 1G, compare, 19–40 min). We estimate that myosin II-dependent forward translocation of the tip of the cell rear initiated toward the end of cell rear formation (Figures 1B and 1D, compare white arrowheads). With time, these dual effects manifested as a long, drawn-out cell tail (Figure 1F) (Movie S1, clip 4) and similar to when myosin II was blocked in a separate population of migrating cells (Figure S2) and polarity gradually destabilized (Figure 1F).

### Temporal Analysis Two of Myosin II Contractility Function

We next identified actin filament organization directly in live polarizing cells expressing GFP or RFP-actin (Figure 2). For more than 90% of cells expressing RFP-actin and treated early with blebbistatin prior to actin bundle alignment, correct organization of actomyosin II then failed; bundles either did not form or did not align during the course of timed observation (Figure 2B), compared to similarly

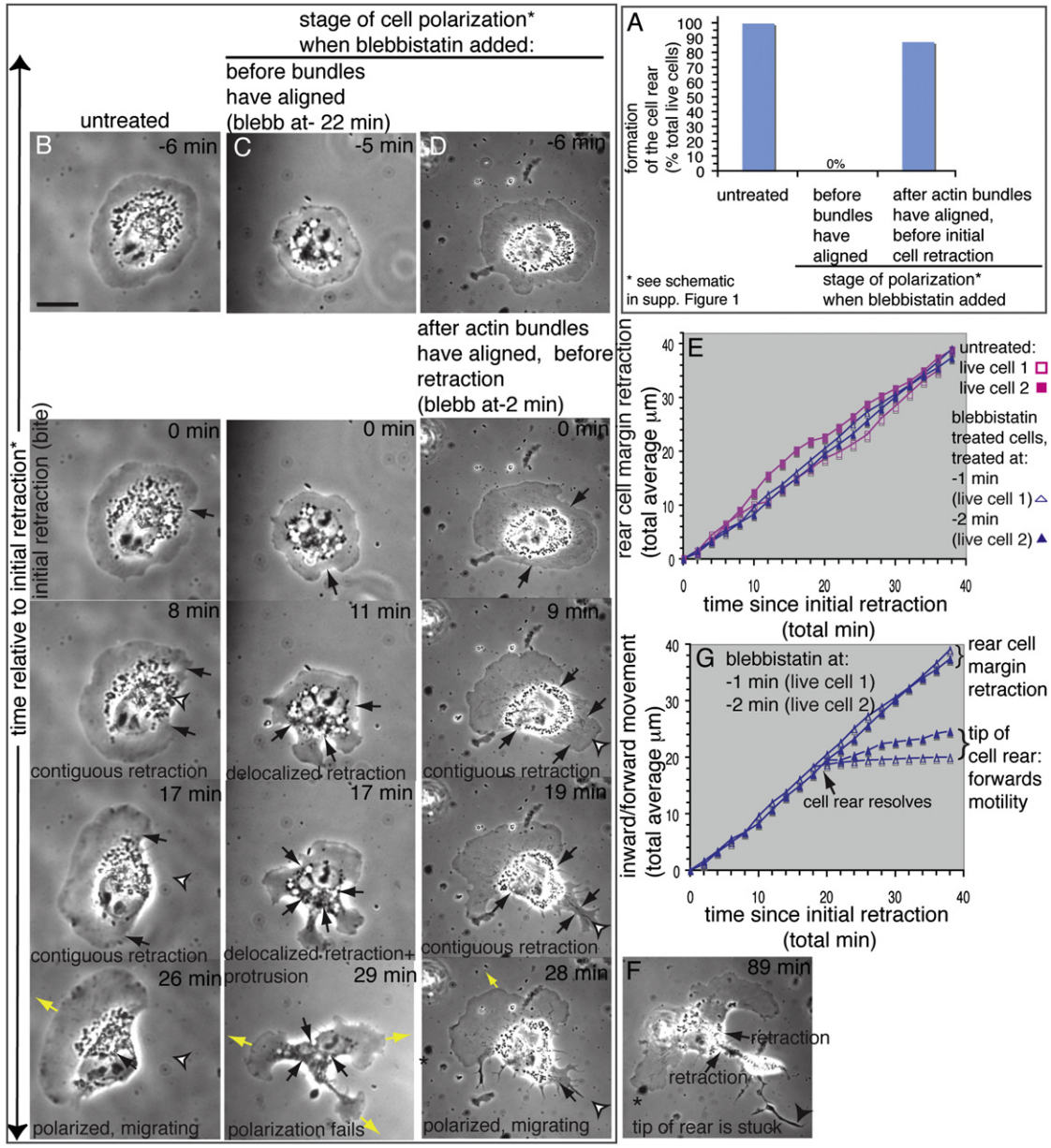


Figure 1. Myosin II-Based Force Is Required for Cell Polarity but Does Not Power Initial Cell Rear Retraction

Live polarizing cells were either left untreated (A, B, and E), or treated with 100 μM (±) (50 μM active concentration) blebbistatin either before (A and C) or after (A and D–G) the expected formation of aligned actomyosin II filament bundles within cells and prior to cell margin retraction and cells imaged live by time-lapse microscopy during (A–E and G) and after cell polarization (E–G).

(A) Quantification of formation of the cell rear, n = 8–16 live cells per condition.

(B–D and F) Still images from time-lapse movies: a single yellow arrow (B and D) indicates the cell front in polarized cells; multiple yellow arrows (C) indicate delocalized cell protrusions; black arrows denote a single, contiguous cell rear (B and D) or delocalized cell retractions (C). Black arrowhead (F) indicates the "stuck" tip of the cell rear that initiated at the beginning of cell migration. White arrowhead (B and D) is the position of the midpoint of the cell rear as it resolved. Asterisk (D and F) is a fiduciary mark on the coverslip; the stage is moved between (D) and (F) as the same cell moves out of the original field of view.

(E) Quantification of inward and/or forward movement of the rear cell margin for two untreated cells (pink) and two cells that were treated with blebbistatin immediately after formation of oriented actomyosin II bundles (blue).

(G) For the two blebbistatin-treated cells in (E), quantification of cell margin retraction at two spatial locations on the same rear cell margin during initial polarization and then during cell migration. Note that myosin II contractility is required for cell polarization as expected (A and C) yet does not establish the cell rear (A, D, and E). Time (B–G) is relative to initial cell margin retraction ("bite"). See also Movie S1. Scale bar in (B) represents 20 μm (B–D and F).

timed untreated cells where actin bundles both formed and oriented (Figure 2A). Consistent with other reports [13–18, 26], cells with incorrect actin organization induced by blocking myosin II contractility also failed to polarize, and these cells did not form a single cell rear (Figure 2B). We obtained similar results with Y27632 (Figure 2C), which blocks Rho kinase-dependent activation of myosin II contractility.

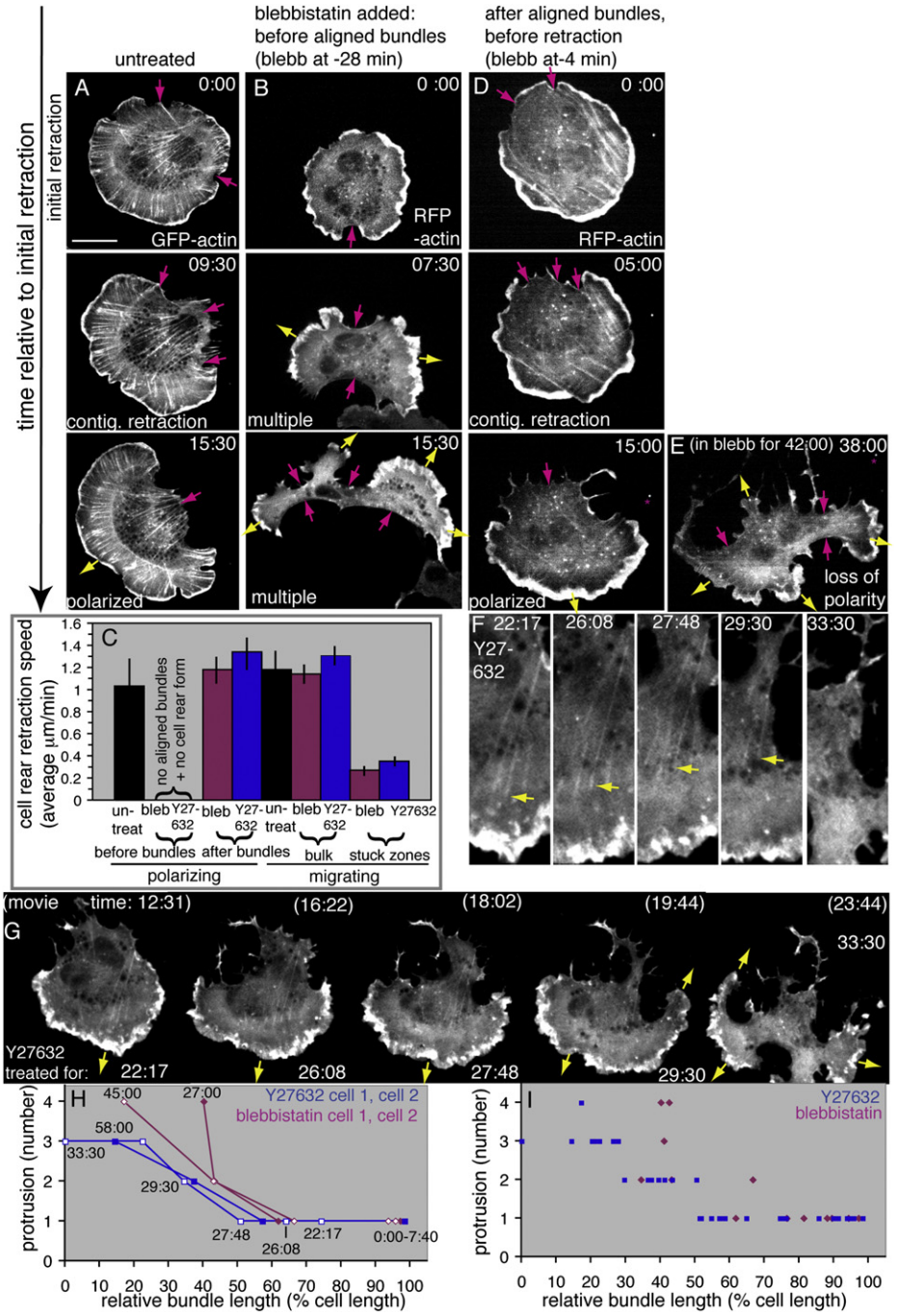


Figure 2. Cell Rear Retraction Forms the Cell Rear in Myosin II-Inhibited Cells with Correct Actin Organization

Actin organization in live polarizing and migrating cells expressing GFP-actin and left untreated (A and C) or RFP-actin (B–E, H, and I) and treated with 100 μM (±) blebbistatin, either before (B and C) or after (C–E, H, and I) actomyosin II filament bundles had aligned in cells and both blebbistatin treatments prior to cell margin retraction or expressing GFP-actin and treated with 10–20 μM Y27632 (C and F–I) directly visualized by spinning disk confocal microscopy.

(A, B, D, and E) Still images from time-lapse movies: a single yellow arrow (A and D) indicates the cell front in polarized cells; multiple yellow arrows (B and E) indicate delocalized cell protrusions; pink arrows denote the cell rear (A and D) or delocalized cell retractions (B and E). Asterisk (D and E) is a fiduciary mark on the coverslip; the stage is moved between (D) and (E) as the same cell moves out of the original field of view. In the same myosin II-inhibited cell (D and G), cell polarity is initially established (D and G), but not maintained (E and G).

(C) Average speed of initial cell rear retraction in polarizing cells and ongoing rear retraction once migration was established. Treatments are before or after formation of aligned actomyosin II bundles. n = 10–24 live cells per condition. Values are mean ± SEM (F–I) Analysis of relative actin bundle length and loss of polarity in myosin II-inhibited migrating cells: cell regions in (F) are aligned at the cell front and are enlargements of (G); yellow arrow in (F) denotes progressive reduction in actin bundle length; and yellow arrows in (G) indicate cell protrusions; (H) is quantification of loss of polarity as a function of relative bundle length for four separate cells with time—open blue squares are the cell shown in (F) and (G)—and (I) for all cells in the population. Time in (A)–(E) is relative to initial cell margin retraction ("bite"). Times in brackets in (G) are total elapsed time since starting filming, times without brackets in (F) and (G) are total time treated with Y27632. Times (H) are total time of drug treatment at the start and end of the analysis of each cell, except for the cell in (F and G), which also indicates intermediate times. See also Movie S2. Scale bar in (A) represents 20 μm (A, B, D, E, and G); scale bar in (A) represents 10 μm (F).

In contrast, in cells where myosin II-based force was inhibited with blebbistatin or Y27632 after actomyosin II bundles had aligned in RFP-actin or GFP-actin expressing cells respectively, cell margin retraction to form the cell rear and establishment of polarity was similar to untreated cells, occurring at a similar speed (Figures 2C and 2D; Movie S2). In treated cells, oriented actin bundles were fairly stable during establishment of polarity (Figure 2D; Movie S2). However, once cell symmetry was broken and cells started to migrate, polarity was not then maintained, which appeared linked with gradual loss of actin bundle organization (Figure 2E; Movie S2). These data (Figure 2) are very consistent with similarly timed inhibition of myosin II in Figure 1.

### Spatiotemporal Analysis of Maintenance of Cell Polarity

We hypothesized, based on our previous findings [21, 23, 27], that myosin II contractility maintains polarity by restricting a single protrusion at one end of the bundles. Note that the cell front can transiently (in untreated cells) or sometimes (in myosin II-inhibited cells) split into two zones, which are counted as one protrusion if each is moving with the same vector $\pm 30^{\circ}$.

When myosin II contractility was inhibited with either blebbistatin or Y27632, we found that existing actin filament bundles progressively spanned less of the cell (Figures 2F–2I) through lack of rebiogenesis as the cell moved [27]. We observed a critical length distribution: above about 50% actin bundle length as a proportion of cell length (see Supplemental Experimental Procedures), cells maintained a single protrusion at the cell front (Figures 2F and 2G, 22:17–27:48) (Figures 2H and 2I; Movie S2). Below about 50%, the numbers of protrusions greater than $\pm 30^{\circ}$ to each other increased above 1, and thus cell polarity was lost (Figures 2F and 2G; 29:30–33:30) (Figures 2H and 2I; Movie S2). Increase in protrusion number was inversely proportional to relative actin bundle length (Figures 2H and 2I; Movie S2). As expected [27], reduction in relative actin bundle length (Figure 2F), left a zone progressively bare of actomyosin II bundles from the front to the back of the cell (Figure 2F). Delocalized protrusions then initiated from the "bare zones" to trigger loss of polarity (Figure 2G, 29:30–33:30) perhaps due to release of contractility-dependent inhibition of cell protrusion [6] or inward plasma membrane tension force [28]. We suggest that myosin II maintains polarity in a spatial loop: graded polarity actomyosin II bundles initially establish [21] and maintain [23] a single cell front at one end of the bundles and then myosin II within the cell front in turn produces new bundles [27] to respan the cell (Figure 2F) as the cell moves forward.

### Actin Filament Depolymerization

Actin filament severing and depolymerization and their regulators ADF and cofilin are important for initial establishment and maintenance of cell polarity [21, 29–32]. These previous studies have not focused on cell rear retraction.

Jasplakinolide specifically and rapidly blocks actin filament disassembly [33, 34]. Importantly, we have previously identified that actin filament depolymerization is required earlier in cell polarization for promoting alignment of actomyosin II bundles [21]. To test for any additional, later role for actin depolymerization in directly powering cell margin retraction, we were therefore very careful to only add jasplakinolide to cells once actin filament bundles had oriented.

Strikingly, acute application of jasplakinolide to cells with aligned actin filament bundles just prior to expected cell margin retraction blocked cell margin retraction; no breaking of cell symmetry occurred up to a total of around 20 min observation in drug (Figures 3A and 3C) (Movie S3, clip 2), approximately 4-fold longer than the time it takes untreated cells to initiate retraction once bundles are aligned (Movie S3, clip 1) [21]. Further, application of jasplakinolide to cells that had just initiated cell margin retraction also prevented any further cell margin retraction (Figures 3B and 3C) (Movie S3, clip 3) observed for up to about 20 min, a time by which in control cells (Figure 1B; Figure 2A; Figure 4A) the cell rear would have fully formed. Overall, cell rear retraction was inhibited in 13/14 cells treated with jasplakinolide. In treated cells, cell polarization failed as expected (Figure 3D). Oriented actin filament bundles appeared intact during jasplakinolide treatment (Figures 3A and 3B). Further, acute jasplakinolide treatment of a separate population of migrating cells also rapidly blocked within 1–2 min cell rear retraction (Figure 3C; Movie S3, clip 4). We argue that the jasplakinolide-induced block in cell rear retraction in these experiments was direct (see Supplemental Results).

### Actin Depolymerization within Subdomains of the Cell Rear

The cell rear retracts in progressive domains during the establishment of polarity [21]. In live cells expressing GFP-actin or RFP-actin, intensity of XFP-fluorescence of obvious polymerized actin structures within individual cell rear domains stably reduced by more than half during retraction (Figures 4A–4C and 4E; Movie S4). Minor fluctuations in fluorescence intensity can also occur prior to polarization, but these are not stable and do not lead to a stable break in cell symmetry (Movie S4). As fluorescence intensity decreased, any released monomer must have rapidly diffused away. Thus we conclude that decrease in fluorescence intensity mostly reflects reduction in net actin polymer. Decrease in actin polymer initiated typically 30–60 s prior to that individual rear zone retracting (Figures 4A, 4B, and 4D) (Movie S4) and continued decreasing as the domain retracted (Figures 4A and 4B) with negligible photobleaching [35]. Thus a decrease in net actin polymer within the cell rear is associated with cell rear retraction. This reflects loss of F-actin polymer during growth cone collapse [36].

Actin polymer did not stably decrease within the cell front during the same time that the rear was retracting in the same cell and may have increased slightly (Figures 4A–4C). Blocking myosin II contractility did not affect the reduction in F-actin polymer associated with cell rear retraction (Figure 4C), consistent with myosin II-independent rear retraction during polarization (Figure 1; Figure 2), whereas inhibiting filament depolymerization with jasplakinolide blocked the decrease in actin polymer (Figures 4C and 4E). Strikingly, relative decrease in F-actin polymer within the rear and rear retraction speed were correlated (Figure 4E). In a separate approach, we altered actin filament turnover in cells by expressing mutant ADF and cofilin or its regulators in migrating cells as described [21, 29]; rear retraction speed was proportionally linked to ADF and cofilin activity (Figure 4F). These data are consistent with reports that rate of actin depolymerization [37] and filament turnover [38] are proportional to overall cell speed in other cells. Together, these findings (Figure 3; Figure 4) provide strong evidence for a role for actin-depolymerization-based cell rear retraction in both polarizing and migrating cells.

## Discussion

Here, spatiotemporal analysis of myosin II contractility and actin depolymerization function has allowed dissection of actin-based mechanism for retraction of the cell rear.

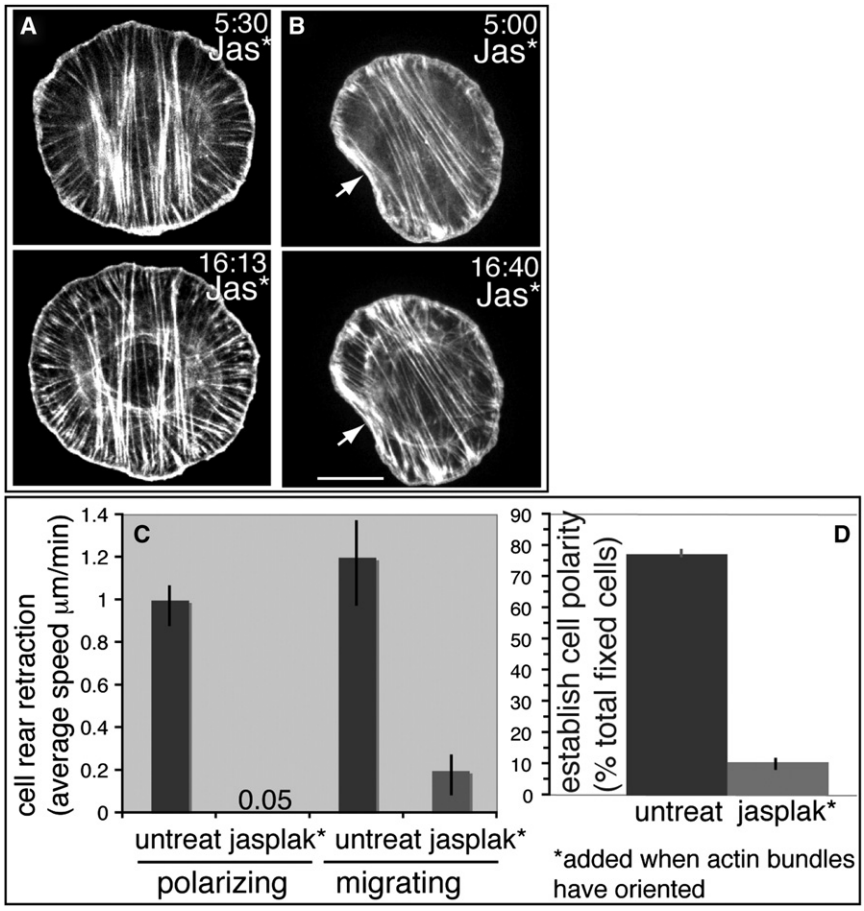


Figure 3. Inhibition of Actin Filament Depolymerization Blocks Cell Rear Retraction in Cells with Correctly Organized Actin

Polarizing cells expressing GFP-actin (A–C) were treated with 0.5 μM jasplakinolide (A–D) after actomyosin II bundles had aligned, left untreated (C–D), or migrating cells without expression vectors (C) were treated with 1 μM jasplakinolide during locomotion.

(A and B) Live cells. A cell treated with jasplakinolide just prior to (A) or immediately at the start of (B) cell margin retraction is shown. Time (A and B) is total time of jasplakinolide treatment.

(C) Live cells. Quantification of cell margin retraction speed, n = 13–14 live cells per condition are shown. Values are mean ± SEM.

(D) Fixed cells. Establishment of polarity in cells with aligned actin bundles and then treated with jasplakinolide for 25 min (n = 300 cells each condition, three repeat experiments) and then fixed is shown. See also Movie S3.

Scale bar in (B) represents 14 μm (A and B).

Our evidence is that actin depolymerization directly powers cell margin retraction to form the cell rear and break cell symmetry in fibroblasts. Thus, remarkably, actin depolymerization may generate motile force similar to that described for unrelated major sperm protein polymer disassembly in Ascaris sperm cells [19, 20]. Further, the actin depolymerization-based force is not directly augmented by myosin II-contractility at this step. We note, in other cells, that actin depolymerization may [34, 37, 39–42] or may not [34, 37] depend on myosin II contractility.

Clearly myosin II contractility has an important earlier function in building correctly organized actomyosin II bundles that are needed to subsequently polarize cells, consistent with data from other studies [13–18]. However, these bundles, at least in polarizing fibroblasts, do not directly power initial retraction; we presume instead that they target spatial activation of actin depolymerization at the cell rear.

On the other hand, once cells have polarized, both actin depolymerization and myosin II contribute mechanical force acting within at least partially distinct spatial zones to pull the cell rear forward during subsequent migration. We speculate that observed myosin II-driven forward movement of the tip of the cell rear corresponds to zones of stronger cellular adhesion, whereas actin depolymerization-based retraction of the bulk cell rear and sides occur within domains of weaker cellular attachments. We predict, based on similar phenotypes [6–9] that in other fibroblast or fibroblast-like cells, the relative importance of actin depolymerization-based and myosin II-based retraction force will also partition spatially along the rear cell margin during migration. In dictyostelium and leucocytes, where rear retraction can occur in the absence of myosin II contractility in some conditions [1] [2], we propose that actin depolymerization powers forward movement of the cell rear; perhaps driving deadhesion of weaker attachments. In support of these notions, we note that ADF and cofilin regulate a subset of cellular adhesions [43], and the relative importance of myosin II contractility for rear retraction in more weakly attached cells increases with increasing cell adhesion [1]. Clearly, a spatiotemporal analysis of myosin II and actin depolymerization function for rear retraction in other cell types will help distinguish precise mechanisms more broadly among different cell types.

We provide conclusive evidence that myosin II-contractility does not directly retract the cell rear in polarizing fibroblasts. We identify a direct role for myosin II in biogenesis of aligned actomyosin II bundles and in specifying their length, thereby maintaining polarity by restricting cell protrusion to the front of the cell. Actin filament depolymerization-based force with no direct requirement for myosin II contractility powers cell rear retraction to break cell symmetry and trigger migration.

### Experimental Procedures

All experimental procedures were performed as previously described [21]. In short, to synchronize cell polarization, primary chick heart fibroblasts were isolated and then pretreated for 1 hr with 1–5 μM latrunculin-A and then latrunculin-A was washed out of cells [21]. Latrunculin-A is thoroughly washed out of cells prior to any subsequent treatment of cells with other cytoskeleton inhibitors. Please see Supplemental Experimental Procedures for detailed information on how stages of cell polarization (Figure S1) were subsequently identified and targeted with 10–20 μm Y27632, 100 μm (±) (50 μm active concentration) blebbistatin or 0.5–1 μM jasplakinolide, and for cell retraction and actin polymer measurements in live cells.

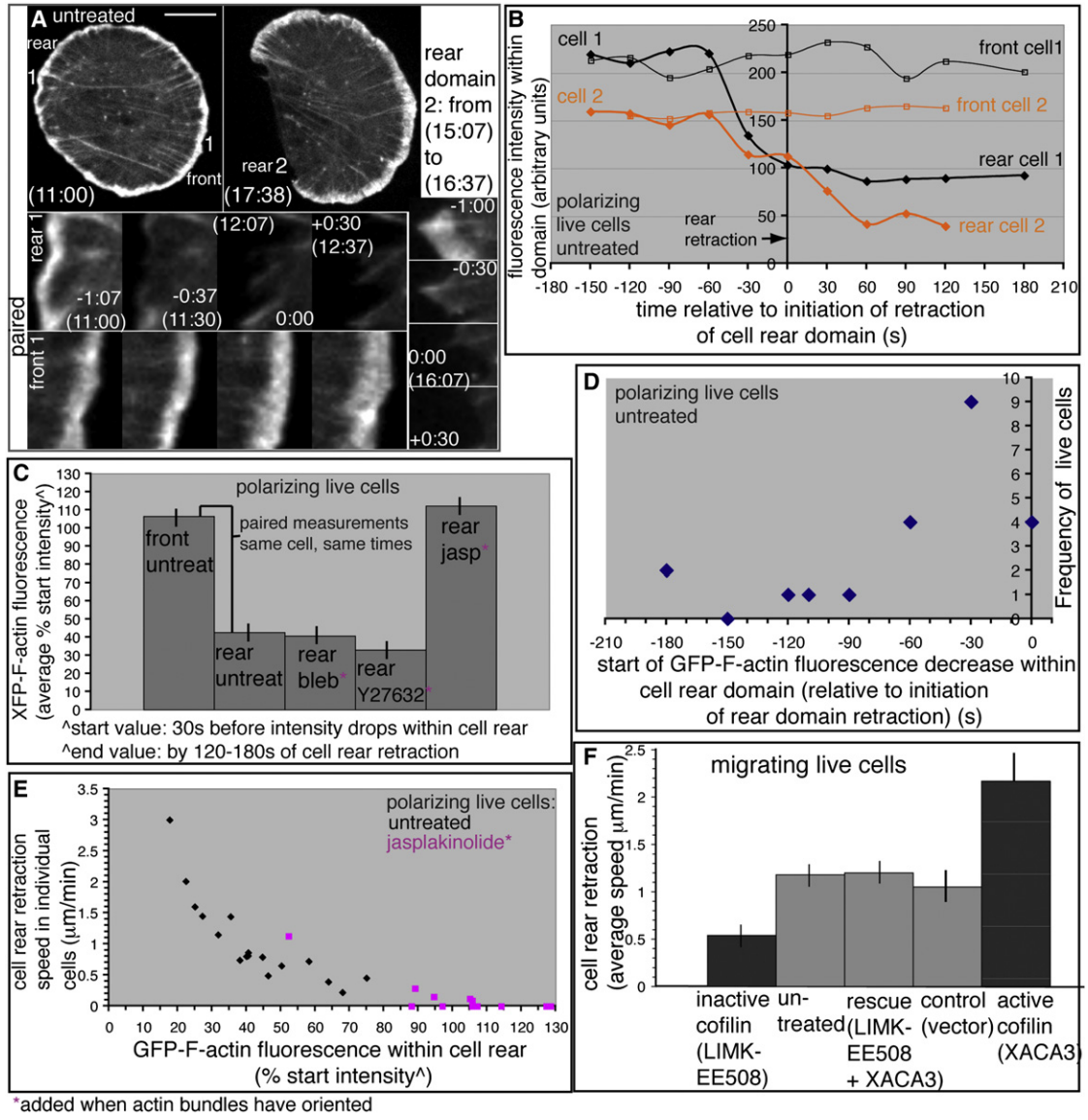


Figure 4. Loss of F-actin Polymer within the Cell Rear Is Coupled to Cell Rear Retraction

Polarizing cells expressing GFP-actin (A–E) or RFP-actin (C) were treated with either 0.5 μM jasplakinolide (C and E) or inhibitors of myosin II (C), all treatments after actomyosin II bundles had aligned, left untreated (A–E), or the proportion of active ADF and cofilin was modulated in migrating cells by expressing either constitutive active LIMK (LIMK-EE508) only, nonphosphorylatable active cofilin (XACA3) only, or cells rescued (LIMK-EE508 and XACA3 together) (F).

(A) Polarizing untreated cell. GFP-F-actin fluorescence intensity within the cell rear and cell front: rear domain 1 is the initial cell retraction that forms the "bite," and rear domain 2 is a retraction that occurs during elaboration of the cell rear. Time in brackets is total time from the beginning of filming. Time without brackets is relative to rear retraction (set to zero) of each given subdomain.

(B) Polarizing untreated cells. GFP-F-actin fluorescence intensity measured for two pairs of cell rear and cell front domains during the retraction phase of polarization is shown.

(C) Polarizing cells, various conditions. Average relative XFP-actin fluorescence intensity, n = 9–18 live cells per condition is shown. Values are mean ± SEM.

(D) Polarizing untreated cells. Frequency of when decrease in GFP-F-actin fluorescence intensity occurs is shown.

(E) Polarizing untreated and jasplakinolide-treated cells. Cell rear retraction speed and relative GFP-F-actin fluorescence are correlated. The single, minority cell where retraction speed was less affected by jasplakinolide was excluded from calculations of averages shown in (C).

(F) Cell rear retraction speed in migrating cells in which the proportion of active ADF and cofilin is modulated, n = 8–11 average values in individual cells per condition. Values are mean ± SEM. See also Movie S4.

Scale bar in (A) represents 14 μm; scale bar in (A) represents 4.5 μm (A, enlargements).

## Supplemental Information

Supplemental Information includes two figures, Supplemental Experimental Procedures, and four movies and can be found with this article online at doi:10.1016/j.cub.2011.11.006.

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